**In the Claims**

This listing of claims will replace all prior versions, and listings, of claims in the application:

1. (Currently Amended) A method for analyzing, by means of mass spectrometry, the C-terminal amino acid sequence of a peptide to be examined, which method comprises the following steps:

a step of preparing a mixture containing a series of reaction products that are obtained from the peptide to be examined by releasing the C-terminal amino acids successively by chemical means,

a step of analyzing the differences in molecular weight between said series of reaction products and the original peptide by means of mass spectrometry to measure the decreases in molecular weight associated with the successive release of the C-terminal amino acids, and

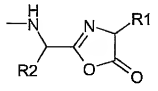
a step of identifying a series of the amino acids removed successively, based on a series of the measured decreases in molecular weight and arranging them from the C-terminus to obtain the information of the C-terminal amino acid sequence of the peptide,

wherein said process for releasing the C-terminal amino acids successively comprises at least the following steps:

a pretreatment step for providing the protection by means of N-acylation, in which N-acylation with the acyl group derived from the alkanoic acid anhydride is applied to the N-terminal amino group of the peptide as well as to the amino group on the side chain of the lysine residue which may be included in the peptide,

a step of allowing an alkanoic acid anhydride to act on the dry sample of said peptide to be examined after N-acylation protection in the presence of a catalytic amount of a perfluoroalkanoic acid to release the C-terminal amino acids in association with a process that at

the C-terminus of the peptide, the formation of a 5-oxazolone structure represented by the following general formula (III):




(III)

wherein R1 is a side chain of the C-terminal amino acid of the peptide and R2 is a side chain of the amino acid residue positioned just before the C-terminal amino acid, is followed by the cleavage of the 5-oxazolone ring, and

a hydrolysis treatment step which comprises applying, to a mixture containing a series of reaction products obtained in said step of releasing the C-terminal amino acids successively, a post-treatment of removing said remaining alkanoic acid anhydride and perfluoroalkanoic acid therefrom, and then allowing water molecules to act thereto in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound to give rise to a hydrolysis reaction,

wherein said step of measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids employs a technique which comprises:

allowing trypsin to act on the sample in a buffer solution to carry out the treatment for the enzymatic digestion specific to trypsin of said peptide chain which holds N-acylation protection as for the N-terminal amino group of the peptide chain as well as to the amino group on the side chain of the lysine residue that may be contained in the peptide chain, and thereby, conducting

selective cleavage of the C-terminal side peptide bond of each arginine residue that present in the peptide chain to complete peptide fragmentization,

applying a desalting treatment to remove the buffer solution component, followed by recovering and drying the peptide fragments after the digestion treatment by trypsin,

next ~~to that~~, conducting, as for the dried mixture containing said peptide fragments recovered after the digestion treatment by trypsin, molecular weight measurement for the cationic species of $(M+H)^+$ as well as molecular weight measurement for the anionic species of $(M-H)^-$ both of which are generated from the ionization treatment by means of MALDI-TOF-MS ~~mass spectrometry~~,

with respect to the corresponding mass spectra of the ion species, which are measured in said molecular weight measurement for the cationic species of $(M+H)^+$ as well as molecular weight measurement for the anionic species of $(M-H)^-$,

judging that the peaks of the peptide fragments each having an arginine residue at the C-terminus, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for the cationic species of $(M+H)^+$ is relatively larger in comparison with the intensity in the molecular weight measurement for the anionic species of $(M-H)^-$, and judging that the peaks of the C-terminal peptide fragment derived from the original peptide and the C-terminal peptide fragments derived from a series of the reaction products that are obtained by successive release of the C-terminal amino acids, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for the anionic species of $(M-H)^-$ is relatively larger in comparison with the intensity in the molecular weight measurement for the cationic species of $(M+H)^+$, and

based on a series of the peaks that gives a relatively larger intensity in the molecular weight measurement for the anionic species of $(M-H)^-$, measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids;

wherein the step of analysis of the spectra, in which the range to be analyzed for the analysis operation of spectra is selected within m/z value of 4,000 or less, comprises the following Steps 1 to 9:

(Step 1) a step for identification of internal standard peaks derived from trypsin, which comprises:

with respect to the peptide fragments derived from the autolysis of trypsin having a known molecular weight, which are concomitant with the digestion treatment with trypsin used for peptide fragmentization, and incorporated into the dry mixture containing the peptide fragments,

identifying the peaks of the cationic species of $(M+H)^+$ due to the peptide fragments derived from trypsin autolysis, in a m/z value range of 4,000 to 500 of the result of the molecular weight measurements for the cationic species of $(M+H)^+$,

then, identifying the peaks of the corresponding anionic species of $(M-H)^-$ due to the peptide fragments resulting from trypsin autolysis, in a m/z range of 4,000 to 500 of the result of the molecular weight measurements for the anionic species of $(M-H)^-$;

(Step 2) a step for identification of major ion species peaks, which comprises:

excluding said peaks assigned for the cationic species peaks derived from trypsin from the result of molecular weight measurements for cationic species, identifying the highest peak of cationic species having the highest peak intensity, in a m/z value range of 4,000 to 500, and by using the peak intensity of the highest peak of cationic species as a basis, selecting peaks of

cationic species each having a peak intensity of 1/40 or more relative to the basis to make up the first group of cationic species peaks therewith,

next to that, excluding said peaks assigned for the anionic species peaks derived from trypsin from the result of molecular weight measurements for anionic species, identifying the highest peak of anionic species having the highest peak intensity, in a m/z value range of 4,000 to 500, and by using the peak intensity of the highest peak of anionic species as a basis, selecting anionic species peaks each having a peak intensity of 1/40 or more relative to the basis to make up the first group of anionic species peaks therewith,

(Step 3) a step for identification of counter ion species peaks for the major ion species peaks, which comprises:

identifying, in the result of the molecular weight measurements for anionic species, peaks due to anionic species each corresponding to each peak of said first group of cationic species peaks to make up the second group of anionic species peaks therewith,

next to that, identifying, in the result of the molecular weight measurements for cationic species, peaks due to cationic species each corresponding to each peak of said first group of anionic species peaks to make up the second group of cationic species peaks therewith,

(Step 4) a step for identification of major ion species peaks having significant counter-ionic species, which comprises:

making up the overlapping group between the first group of anionic species peaks and the second group of anionic species peaks to define it as the third group of anionic species peaks, and also making up the sum group of the first group of anionic species peaks and the second group of anionic species peaks to define it as the fourth group of anionic species peaks,

next to that, making up the overlapping group between the first group of cationic species peaks and the second group of cationic species peaks to define it as the third group of cationic species peaks, and also making up the sum group of the first group of cationic species peaks and the second group of cationic species peaks to define it as the fourth group of cationic species peaks,

with respect to each cationic species peak corresponding to each peak of said third group of anionic species peaks, calculating the relative peak intensity on the basis of said peak intensity of the highest cationic species peak identified at the Step 2 and, with respect to each anionic species peak of said third group of anionic species peaks, calculating the relative peak intensity on basis of said peak intensity of the highest anionic species peak identified at the Step 2, and comparing the two relative peak intensities with each other,

identifying those corresponding cationic species peaks each having a relative intensity which is 3/2 or more relative to that of the peak of the third group of anionic species peaks to make up the fifth group of cationic species peaks therewith,

meanwhile, identifying those anionic species peaks each having a relative intensity which is 3/2 or more relative to that of the corresponding cationic species peaks to make up the fifth group of anionic species peaks therewith,

(Step 5) a step for identification of major ion species peaks caused by peptide fragments derived from target peptide to be analyzed, which comprises:

based on the m/z value of each cationic species peak of the fourth group of cationic species peaks, calculating the differences in the m/z value between each adjacent peaks thereof,

meanwhile, based on the m/z value of each anionic species peak of the fourth group of anionic species peaks, calculating the differences in the m/z value between each adjacent peaks thereof,

with respect to each peak of the fifth group of cationic species peaks, examining said peak as to the following criteria:

(5a-1) a cationic species peak having a m/z value smaller than the m/z value of said peak by the molecular weight of 18 corresponding to loss of one water molecule is present in the fifth group of cationic species peaks;

(5a-2) a cationic species peak having a m/z value larger than the m/z value of said peak by the molecular weight excess equivalent to the formula weight of the acyl group used for said N-acylation protection is present in the fifth group of cationic species peaks; and

(5a-3) a cationic species peak having a m/z value larger than the m/z value of said peak by the combination of the molecular weight decrease of 18 corresponding to loss of one water molecule and excess equivalent to the formula weight of the acyl group used for said N-acylation protection is present in the fifth group of cationic species peaks;

to select those cationic species peaks each satisfying at least one of said requirements (5a-1) to (5a-3), and then making up the sixth group of cationic species peaks therewith,

meanwhile, with respect to each peak of the fifth group of anionic species peaks, examining said peak as to the following criteria:

(5b-1) an anionic species peak having a m/z value smaller than the m/z value of said peak by the molecular weight of 18 corresponding to loss of one water molecule is present in the fifth group of anionic species peaks;

(5b-2) an anionic species peak having a m/z value larger than the m/z value of said peak by the molecular weight excess equivalent to the formula weight of the acyl group used for said N-acylation protection is present in the fifth group of anionic species peaks; and

(5b-3) an anionic species peak having a m/z value larger than the m/z value of said peak by the combination of the molecular weight decrease of 18 corresponding to loss of one water molecule and excess equivalent to the formula weight of the acyl group used for said N-acylation protection is present in the fifth group of anionic species peaks;

to select those anionic species peaks each satisfying at least one of said requirements (5b-1) to (5b-3), and then making up the sixth group of anionic species peaks therewith,

judging that the sixth group of cationic species peaks selected thereby are a group of cationic species peaks caused by peptide fragments derived from the target peptide to be analyzed, and judging also that the six group of anionic species peaks selected thereby are a group of anionic species peaks caused by peptide fragments derived from the target peptide to be analyzed,

(Step 6) a step for identification of ion species peaks of peptide fragments per se derived from target peptide to be analyzed, which comprises:

with respect to each peak of the sixth group of cationic species peaks, in comparison with the relative intensities of its accompanying cationic species peaks that are relevant to at least one of said relationships (5a-1) to (5a-3) for said peak, selecting peaks whose relative intensities are superior to their accompanying cationic species peaks, and then from the group of the selected peaks, further selecting peaks which is not one of accompanying cationic species peaks that are relevant to at least one of said relationships (5a-1) to (5a-3) for other peak included in said group

of the selected peaks and have an inferior relative intensity to other peak, and making up the seventh group of cationic species peaks therewith,

meanwhile, with respect to each peak of the sixth group of anionic species peaks, in comparison with the relative intensities of its accompanying anionic species peaks that are relevant to at least one of said relationships (5b-1) to (5b-3) for said peak, selecting peaks whose relative intensities are superior to their accompanying anionic species peaks, and then from the group of the selected peaks, further selecting peaks which is not one of accompanying anionic species peaks that are relevant to at least one of said relationships (5b-1) to (5b-3) for other peak included in said group of the selected peaks and have an inferior relative intensity to other peak, and making up the seventh group of anionic species peaks therewith,

judging that the seventh group of cationic species peaks are a group of cationic species peaks caused by peptide fragments per se derived from the target peptide to be analyzed, and judging also that the seventh group of anionic species peaks are a group of anionic species groups caused by peptide fragments per se derived from the target peptide to be analyzed,

(Step 7) a step for identification of peptide fragments each having arginine at the C-terminus of its peptide chain, produced by the digestion treatment by trypsin, which comprises:

selecting each anionic species peak which corresponds to each cationic species peak of the seventh group of cationic species peaks, from the peaks being present in the fourth group of anionic species peaks, to make up the eighth group of anionic species peaks therewith,

with respect to each peak of the eighth group of anionic species peaks, selecting group of anionic species peaks of which a m/z value difference from the fiducial m/z value of the said anionic species peak is found within the range of less than 200, based on set of the m/z value

differences between adjacent peaks that are calculated in the Step 5, from the peaks being present in the fourth group of anionic species peaks, and

confirming that there is not present, in the thus-selected groups, any peak whose m/z value difference between those peaks is equivalent to the formula weight of natural chain a-amino acid residue: -NH-CH(R)-CO- (R is a side chain of said amino acid residue) or of a-amino acid residue protected by acylation wherein the hydroxy group or amino group of its side chain is modified by substitution with the acyl group used in said N-acylation protection, and thus judging that said eighth group of anionic species peaks are the group of anionic species peaks from peptide fragments each having arginine at the C-terminus of its peptide chain, which are derived from the target peptide to be examined and produced by the treatment for digestion by trypsin,

(Step 8) a step for identification of group of C-terminal side peptide fragments that are produced from target peptide and a series of reaction products thereof by the treatment for digestion by trypsin, which comprises:

with respect to each anionic species peak of the seventh group of anionic species peaks, selecting group of anionic species peaks of which a m/z value difference from the fiducial m/z value of the said anionic species peak is found within the range of less than 200, based on set of the m/z value differences between adjacent peaks that are calculated in the Step 5, from the peaks being present in the fourth group of anionic species peaks, and

identifying those anionic species peaks being included in the seventh group of anionic species group, for which there is present, in the thus-selected groups, a peak whose m/z value difference between those peaks is equivalent to the formula weight of natural chain a-amino acid residue: -NH-CH(R)-CO- (R is a side chain of said amino acid residue) or of a-amino acid

residue protected by acylation wherein the hydroxy group or amino group of its side chain is modified by substitution with the acyl group used in said N-acylation protection, and then making up the ninth group of anionic species peaks therewith,

forming the summed-up group of each anionic species peak of the ninth group of anionic species peaks and each of said anionic species peaks being present in the fourth group of anionic species peaks whose m/z value difference between those peaks has been confirmed, in said operation of identification, to be equivalent to the formula weight of amino acid residue, and then defining the group as the tenth group of anionic species peaks, from said largest m/z peak,

selecting, in the tenth group of anionic species peaks, an anionic species peak having the largest m/z value, successively identifying, from the tenth group of anionic species peaks, a series of anionic species peaks each having a m/z value difference between peaks that is equal to the formula weight of amino acid residue, by using, as the finducial point, the m/z value which the anionic species peak with the largest m/z value shows, and then judging that the series of thus-identified peaks as the group consisting of the anionic species peak of C-terminal peptide fragment derived from the original peptide and the anionic species peaks of C-terminal peptides derived from a series of reaction products that are obtained by successive release of C-terminal amino acids of original peptide, which fragments are all produced by the treatment for digestion by trypsin, and

(Step 9) a step for assignment of C-terminal amino acid sequence, which comprises:

according to a series of said formula weights of amino acid residues that are corresponding to the m/z differences between the anionic species peaks, which have been sequentially assigned in Step 8, based on the identified group consisting of the anionic species peaks of C-terminal peptide fragments that are derived from the original peptide and a series of

reaction products resulted from successive release of C-terminal amino acids, which fragments are all produced by the treatment for digestion by trypsin, identifying the sequence of partial amino acids which have been released successively from the C-terminus thereof.

2. (Original) A method for analysis claimed in Claim 1,

wherein, after Step 1 being the step for identification of internal standard peaks derived from trypsin, there is employed a step for noise removal and smoothening treatment, which comprises:

with respect to each cationic species peak of the peptide fragments derived from trypsin autolysis, identified in the result of the molecular weight measurements for cationic species, determining its peak m/z value and calculating its apparent full-width of half maximum,

by using said apparent full-width of half maximum calculated as the datum width, conducting, for the spectra of molecular weight measurement for cationic species peak, a treatment of removing noise peaks each showing an apparent full-width of half maximum which is 1/4 or less of the datum width,

then, conducting, for the spectra after the treatment for noise removal, a smoothing treatment such that the asymmetry of peak shape and the integrated intensity of peak as for each cationic species peak of the peptide fragments derived from trypsin autolysis can be well-retained, which are evaluated based on the determined peak m/z values and the two m/z values used in calculation of said apparent full-width of half maximum and,

meanwhile, with respect to each anionic species peak of the peptide fragments derived from trypsin autolysis, identified in the result of the molecular weight measurements for anionic species, determining its peak m/z value and calculating its apparent full-width of half maximum,

by using said apparent full-width of half maximum calculated as the datum width, conducting, for the spectra of molecular weight measurement for anionic species peak, a treatment of removing noise peaks each showing an apparent full-width of half maximum which is 1/4 or less of the datum width,

conducting, for said anionic species peak, a treatment of removing noise peaks each having an apparent half width which is 1/4 or less of the above-calculated apparent half width,

then, conducting, for the spectra after the treatment for noise removal, a smoothing treatment such that the asymmetry of peak shape and the integrated intensity of peak as for each anionic species peak of the peptide fragments derived from trypsin autolysis can be well-retained, which are evaluated based on the determined peak m/z values and the two m/z values used in calculation of said apparent full-width of half maximum.

3. (Original) A method for analysis claimed in Claim 1 or 2,

wherein, after Step 1 being the step for identification of internal standard peaks derived from trypsin, there is employed a step for systematic error correction for peak m/z value, which comprises:

with respect to the cationic species peak of each peptide fragment derived from the trypsin autolysis, identified in the result of molecular weight measurements based on cationic species, calculating the m/z value of said cationic species based on the known molecular weight of said peptide fragment, comparing it with the peak m/z value measured therefor on the spectra and, based on their difference, making a correction of systematic error for the m/z value measured in spectra of the molecular weight measurements based on cationic species,

meanwhile, with respect to the anionic species peak of each peptide fragment derived from the trypsin autolysis, identified in the result of molecular weight measurements based on anionic species, calculating the m/z value of said anionic species based on the known molecular weight of said peptide fragment, comparing it with the peak m/z value measured therefor on the spectra and, based on their difference, making a correction of systematic error for the m/z value measured in spectra of the molecular weight measurements based on anionic species.

4. (Currently Amended) A method for analysis claimed in Claim 1,

wherein, in Step 9 being the step for assignment of C-terminal amino acid sequence,

when the assigned sequence of partial amino acids which have been released successively from the C-terminus of original peptide, has arginine as the C-terminal amino acid, there is optionally employed a step for reconfirming the assignment such that its C-terminal fragment is a peptide fragment having arginine at the C-terminus of its peptide chain, which comprises:

with respect to the anionic species peak having the largest m/z value in the tenth group of anionic species peaks, which is used as the ~~finducial~~ fiducial peak for the assignment of partial amino acid sequence, finding, in the result of the molecular weight measurement based on cationic species, a cationic species peak corresponding thereto,

as for the corresponding cationic species peak, selecting group of cationic species peaks of which a m/z value is larger than the fiducial m/z value of the said anionic species peak and the m/z value difference therebetween is found within the range of less than 200, based on set of the m/z value differences between adjacent peaks that are calculated in the Step 5, from the peaks being present in the fourth group of cationic species peaks, and

confirming that there is not present, in the thus-selected groups, any peak whose m/z value difference between those peaks is equivalent to the formula weight of natural chain a-amino acid residue: -NH-CH(R)-CO- (R is a side chain of said amino acid residue) or of a-amino acid residue protected by acylation wherein the hydroxy group or amino group of its side chain is modified by substitution with the acyl group used in said N-acylation protection.

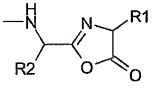
5. (Currently Amended) A method for analysis claimed in Claim 1, wherein the mass spectra used to measure the decreases in molecular weight associated with said successive release of the C-terminal amino acids, are said results of the molecular weight measurements based on the cationic species of $(M+H)^+$ as well as of molecular weight measurements based on the anionic species of $(M-H)^-$, by means of MALDI-TOF-MS.

6. (Currently Amended) A method for analysis claimed in Claim 5,

wherein said process for releasing the C-terminal amino acids successively comprises at least the following steps:

a pretreatment step, for providing the protection by means of N-acylation, of allowing an alkanoic acid anhydride and an alkanoic acid both of vapor phase, which are supplied from a mixture of the alkanoic acid anhydride with a small amount of the alkanoic acid added thereto, to act on a dry sample of said peptide to be examined in a dry atmosphere at a temperature selected in a range of 10°C to 60°C and, thereby, applying, to the N-terminal amino group of the peptide as well as to the amino group on the side chain of the lysine residue which may be included in the peptide, N-acylation by the acyl group derived from the alkanoic acid anhydride,

a step of allowing an alkanoic acid anhydride and a perfluoroalkanoic acid both of vapor phase, which are supplied from a mixture of an alkanoic acid anhydride with a small amount of a perfluoroalkanoic acid added thereto, to act on the dry peptide sample after N-acylation protection in a dry atmosphere at a temperature selected in a range of 15°C to 60°C and, thereby, releasing the C-terminal amino acids successively in association with a process that at the C-terminus of the peptide, the formation of a 5-oxazolone structure represented by the following general formula (III):




(III)

wherein R1 is a side chain of the C-terminal amino acid of the peptide and R2 is a side chain of the amino acid residue positioned just before the C-terminal amino acid, is followed by the cleavage of the 5-oxazolone ring, and

a hydrolysis treatment step which comprises applying, to a mixture containing a series of reaction products obtained in said step of releasing the C-terminal amino acids successively, a post-treatment of removing the remaining alkanoic acid anhydride and perfluoroalkanoic acid in a dry state, and then supplying with a basic nitrogen-containing aromatic compound or a tertiary amine compound and water molecules, all of vapor phase, with use of an aqueous solution dissolving the basic nitrogen-containing, aromatic compound or the tertiary amine compound therein, to allow the water molecules to act on the peptides of the reaction products in the presence of the basic nitrogen-containing organic compound to give rise to a hydrolysis

treatment, and after that conducting the re-dried up treatment by removing, from the mixture containing a series of reaction products, the remaining basic nitrogen-containing organic compound and water molecules to dry up the mixture,

wherein said step of measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids employs a technique which comprises:

allowing trypsin to act on said mixture, after the the re-dried up treatment, containing a series of the reaction products finished by hydrolysis treatment, in a buffer solution, to carry out the treatment for the enzymatic digestion specific to trypsin of said peptide chain which holds N-acylation protection as for the N-terminal amino group of the peptide chain as well as to the amino group on the side chain of the lysine residue that may be contained in the peptide chain, and thereby, conducting selective cleavage of the C-terminal side peptide bond of each arginine residue that present in the peptide chain to complete peptide fragmentization,

applying a desalting treatment to remove the buffer solution component, followed by recovering and drying the peptide fragments after the digestion treatment by trypsin, followed by drying,

next ~~to that~~, conducting, as for the dried mixture containing said peptide fragments recovered after the digestion treatment by trypsin, molecular weight measurement for the cationic species of $(M+H)^+$ as well as molecular weight measurement for the anionic species of $(M-H)^-$, both of which are generated from the ionization treatment, by means of MALDI-TOF-MS.

7. (Currently Amended) A method for analysis claimed in Claim 5,

wherein said process for releasing the C-terminal amino acids successively, as for the sample of the target peptide that has been subjected to separation by gel electrophoresis and is maintained in a state that it is bound on a gel carrier, comprises the following steps:

a step of removing the water solvent impregnated into the gel carrier by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for water, to conduct a dehydration treatment for the gel carrier,

a pretreatment step for the target peptide sample that is still bound on the gel carrier after carrying out said step for dehydration treatment, in which pretreatment step

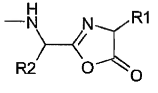
applying N-acylation protection by the acyl group derived from the alkanoic acid constituting said alkanoic acid anhydride, to the N-terminal amino group of the target peptide with use of a solution of an alkanoic acid anhydride dissolved in a dipolar aprotic solvent that is capable of infiltrating into the gel substance and keeping it in a swollen state is conducted by immersing, at a temperature selected in a range of 30°C to 80°C, the gel carrier in the solution of the alkanoic acid anhydride to allow the alkanoic acid anhydride to act on the target peptide sample that is kept in the bound state; and then

removal of said solution is carried out by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for the alkanoic acid anhydride as well as the dipolar aprotic solvent, to conduct termination of the N-acylation reaction and removal of the reaction reagent therefor;

a step of treatment as for the target peptide sample bound on the gel carrier, after the pretreatment step of N-acylation protection, comprising steps of:

immersing, at a temperature selected in a range of 30 °C to 80 °C, the gel carrier in a mixed solution of an alkanoic acid anhydride added with a small amount of a perfluoroalkanoic

acid in relative ratio thereto dissolved in a dipolar aprotic solvent that is capable of infiltrating into the gel substance and keeping it in a swollen state, to allow the alkanoic acid anhydride and the perfluoroalkanoic acid to act on the target peptide sample being kept in the bound state; thereby, successive release of the C-terminal amino acids results from the reaction process with use of the mixed solution in which formed is a 5-oxazolone-ring structure represented by the following general formula (III):




(III)

wherein R1 is a side chain of the C-terminal amino acid of the peptide and R2 is a side chain of the amino acid residue positioned just before the C-terminal amino acid, followed by the cleavage of the 5-oxazolone-ring, and

removing the mixed solution used in the reaction for successive release of C-terminal amino acids, by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for the perfluoroalkanoic acid and the alkanoic acid anhydride as well as the dipolar aprotic solvent, to conduct termination of the releasing reaction and removal of the reaction reagents therefor; and

an additional step for hydrolysis treatment and then redehydration treatment, in which step

the hydrolysis treatment for said mixture comprising a series of reaction products obtained by the reaction for successive release of C-terminal amino acids is conducted by

immersing the gel carrier in an aqueous solution dissolving a basic nitrogen-containing aromatic compound or a tertiary amine compound therein to allow a water molecule to act, in the presence of said basic nitrogen-containing organic compound, on said peptides of the reaction products being still bound on the gel carrier, and then,

the redehydration treatment for the gel carrier is performed by removing said aqueous solution infiltrated into the gel carrier by dilution with use of a polar aprotic solvent having no solvency for the gel substance and having affinity for water; and

wherein said step of measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids employs a method which comprises:

allowing trypsin being soluble in a buffer solution to act on said mixture, after the re-dried up treatment, containing a series of the reaction products finished by hydrolysis treatment to carry out the treatment for the enzymatic digestion specific to trypsin of said peptide chain which holds N-acylation protection as for the N-terminal amino group of the peptide chain as well as to the amino group on the side chain of the lysine residue that may be contained in the peptide chain, and thereby, conducting selective cleavage of the C-terminal side peptide bond of each arginine residue that present in the peptide chain to complete peptide fragmentization,

applying a desalting treatment to remove the buffer solution component, followed by recovering and drying the peptide fragments after the digestion treatment by trypsin, followed by drying,

next ~~to that~~, conducting, as for the dried mixture containing said peptide fragments recovered after the digestion treatment by trypsin, molecular weight measurement for the cationic species of $(M+H)^+$ as well as molecular weight measurement for anionic species of $(M-H)^-$, both of which are generated from the ionization treatment, by means of MALDI-TOF-MS.

8. (Previously Presented) A method for analysis claimed in Claim 6 or 7, wherein, in said combination of a perfluoroalkanoic acid and an alkanoic acid anhydride, used for the formation of 5-oxazolone structure and subsequently for the reaction for release of C-terminal amino acids in association with cleavage of the 5-oxazolone ring, there is used, as the alkanoic acid anhydride, a symmetric anhydride of an alkanoic acid having 2 to 4 carbon atoms.

9. (Previously Presented) A method for analysis claimed in Claim 8, wherein there is used, as the symmetric anhydride of an alkanoic acid of 2 to 4 carbon acids, a symmetric anhydride of a linear-chain alkanoic acid having 2 to 4 carbon atoms.

10. (Original) A method for analysis claimed in Claim 6 or 7, wherein, in said combination of a perfluoroalkanoic acid and an alkanoic acid anhydride, used for the formation of 5-oxazolone structure and subsequently for the reaction for release of C-terminal amino acids in association with cleavage of the 5-oxazolone ring, there is used acetic anhydride as the alkanoic acid anhydride.

11. (Original) A method for analysis claimed in Claim 6 or 7, wherein, in said combination of a perfluoroalkanoic acid and an alkanoic acid anhydride, used for the formation of 5-oxazolone structure and subsequently for the reaction for release of C-terminal amino acids in association with cleavage of the 5-oxazolone ring, there is used, as the perfluoroalkanoic acid, a perfluoroalkanoic acid of which pKa is in a range of 0.3 to 2.5.

12. (Original) A method for analysis claimed in Claim 6 or 7, wherein, in said combination of a perfluoroalkanoic acid and an alkanoic acid anhydride, used for the formation of 5-oxazolone structure and subsequently for the reaction for release of C-terminal amino acids in association with cleavage of the 5-oxazolone ring, there is used, as the perfluoroalkanoic acid, a perfluoroalkanoic acid having 2 to 4 carbon atoms.

13. (Original) A method for analysis claimed in Claim 6 or 7, wherein, in said combination of a perfluoroalkanoic acid and an alkanoic acid anhydride, used for the formation of 5-oxazolone structure and subsequently for the reaction for release of C-terminal amino acids in association with cleavage of the 5-oxazolone ring, the content ratio of the alkanoic acid anhydride and the perfluoroalkanoic acid is selected in a range of 1 to 20 volumes per 100 volumes of the alkanoic acid anhydride.

14. (Original) A method for analysis claimed in Claim 6 or 7, wherein, there is used, as the alkanoic acid anhydride used in said pretreatment step of applying N-acylation protection, a symmetric anhydride of an alkanoic acid having 2 to 4 carbon atoms.

15. (Original) A method for analysis claimed in Claim 14, wherein there is used, as the symmetric anhydride of an alkanoic acid having 2 to 4 carbon atoms, a symmetric anhydride of a linear-chain alkanoic acid having 2 to 4 carbon atoms.

16. (Original) A method for analysis claimed in Claim 6 or 7, wherein, as the alkanoic acid anhydride used in said pretreatment step of applying N-acylation protection, there is used acetic anhydride.

17. (Original) A method for analysis claimed in Claim 6 or 7, wherein there is used acetic anhydride as the alkanoic acid anhydride used in said pretreatment step of applying N-acylation protection and also as the alkanoic acid anhydride employed in the combination of a perfluoroalkanoic acid and an alkanoic acid anhydride, used for the formation of 5-oxazolone structure and subsequently for the reaction for release of C-terminal amino acids in association with the cleavage of the 5-oxazolone ring.

18. (Currently Amended) A method for analyzing, by means of mass spectrometry, the C-terminal amino acid sequence of a peptide to be examined, which method comprises the following steps:

(a) a step of preparing a mixture containing a series of reaction products that are obtained from the peptide to be examined by releasing the C-terminal amino acids successively by chemical means,

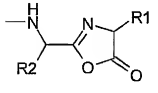
(b) a step of analyzing the differences in molecular weight between said series of reaction products and the original peptide by means of MALDI-TOF-MS ~~mass spectrometry~~ to measure the decreases in molecular weight associated with the successive release of the C-terminal amino acids, and

(c) a step of identifying a series of the amino acids removed successively, based on a series of the measured decreases in molecular weight and arranging them from the C-terminus to obtain the information of the C-terminal amino acid sequence of the peptide,

wherein said process for releasing the C-terminal amino acids successively comprises at least the following steps:

a pretreatment step for providing the protection by means of N-acylation, in which N-acylation with the acyl group derived from the alkanoic acid anhydride is applied to the N-terminal amino group of the peptide as well as to the amino group on the side chain of the lysine residue which may be included in the peptide,

a step of allowing an alkanoic acid anhydride to act on the dry sample of said peptide to be examined after N-acylation protection in the presence of a catalytic amount of a perfluoroalkanoic acid to release the C-terminal amino acids in association with a process that at the C-terminus of the peptide, the formation of a 5-oxazolone structure represented by the following general formula (III):




(III)

wherein R1 is a side chain of the C-terminal amino acid of the peptide and R2 is a side chain of the amino acid residue positioned just before the C-terminal amino acid, is followed by the cleavage of the 5-oxazolone ring, and

a hydrolysis treatment step which comprises applying, to a mixture containing a series of reaction products obtained in said step of releasing the C-terminal amino acids successively, a post-treatment of removing said remaining alkanoic acid anhydride and perfluoroalkanoic acid therefrom, and then allowing water molecules to act thereto in the presence of a catalytic amount of a basic, nitrogen-containing, aromatic compound or a tertiary amine compound to give rise to a hydrolysis reaction,

wherein said step of measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids employs a technique which comprises:

allowing trypsin to act on the sample in a buffer solution to carry out the treatment for the enzymatic digestion specific to trypsin of said peptide chain which holds N-acylation protection as for the N-terminal amino group of the peptide chain as well as to the amino group on the side chain of the lysine residue that may be contained in the peptide chain, and thereby, conducting selective cleavage of the C-terminal side peptide bond of each arginine residue that present in the peptide chain to complete peptide fragmentization,

applying a desalting treatment to remove the buffer solution component, followed by recovering and drying the peptide fragments after the digestion treatment by trypsin,

next ~~to that~~, conducting, as for the dried mixture containing said peptide fragments recovered after the digestion treatment by trypsin, molecular weight measurement for the cationic species of $(M+H)^+$ as well as molecular weight measurement for the anionic species of $(M-H)^-$, both of which are generated from the ionization treatment by means of MALDI-TOF-MS ~~mass spectrometry~~,

with respect to the corresponding mass spectra of the ion species, which are measured in said molecular weight measurement for the cationic species of $(M+H)^+$ as well as molecular weight measurement for the anionic species of $(M-H)^-$,

judging that the peaks of the peptide fragments each having an arginine residue at the C-terminus, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for the cationic species is of $(M+H)^+$ relatively larger in comparison with the intensity in the molecular weight measurement for the anionic species of $(M-H)^-$, and judging that the peaks of the C-terminal peptide fragment derived from the original peptide and the C-terminal peptide fragments derived from a series of the reaction products that are obtained by successive release of the C-terminal amino acids, which fragments are produced by said digestion treatment by trypsin, are peaks that give such intensities that the intensity in the molecular weight measurement for the anionic species of $(M-H)^-$ is relatively larger in comparison with the intensity in the molecular weight measurement for the cationic species of $(M+H)^+$, and

based on a series of the peaks that gives a relatively larger intensity in the molecular weight measurement for the anionic species of $(M-H)^-$, measuring the decreases in molecular weight associated with the successive release of the C-terminal amino acids.